Cell Volume Distribution Pattern Analysis: A Means of Uterine Cancer Detection

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Abstract:

The use of cell volume distribution pattern analysis as a semiautomated means of uterine cancer detection has been evaluated. The results of a study of 339 patients indicate that cell sizing effectively detects preinvasive lesions of the uterus. Compared with the Papanicolaou smear, cell volume distribution pattern analysis is as sensitive as the conventional technic in detecting invasive squamous-cell carcinoma of the cervix. However, for detection of adenocarcinoma of the endocervix and endometrium, cell sizing appears more than twice as sensitive as the conventional Ayre scrape. It was found that *Trichomonas vaginalis* under certain conditions acts as an interfering factor. From *in vitro* studies these conditions were determined and the means of eliminating the organism as a contaminant were considered.

Article:

The control of uterine cancer remains a vexing problem despite the proven reliability of the Papanicolaou smear. One of the difficulties lies in the qualitative nature of exfoliative cytology. Cells are classified not by measurements of cell parameters but by the judgment of experienced cytotechnologists. Their decision is based on a set of morphologic factors which are considered significant in distinguishing normal from malignant cells. It would be of considerable benefit if cell classification could be given a quantitative basis to eliminate the need for detailed visual inspection of each preparation. New quantitative methods in vaginal-cervical cytology must be developed if screening of large segments of the female population for cancer is to become a reality. When one calculates the amount of time necessary to screen hire population segments annually, the formidable nature of the problem becomes apparent.

The present investigation was aimed at developing a new approach to the detection of uterine malignancy. The basic hypothesis of this approach is that there is a distinct difference between cell volumes of the cells exfoliated from the epithelia of normal patients and those desquamated from patients with various genital abnormalities. The previous absence of data concerning differences in total cell volumes has been due mainly to a lack of technology for accurate determinations. With the development of the electronic particle counter, precise quantitative data about the patterns of differential cell volumes in normal and malignant epithelia can now be achieved. The results of an initial study using cell volume distribution pattern analysis suggested that this technic might provide a rapid method for the detection of uterine malignancy.[13]

In the initial study, it was found that *Trichomonas vaginalis* organisms under some conditions had the same volume distribution as the abnormal cells, resulting in false-positive readings. Following the initial study, an additional 339 patients were screened. The analysis and results of the expanded study and a discussion of *T. vaginalis* as an interfering factor are the subject of this report.

Material and Methods

The cell collections for this study were obtained from women who visited the Obstetrics and Gynecology Outpatient Clinics at the University of Wisconsin Medical Center, as well as patients admitted to the Gynecology Service of the Hospital. Since the University of Wisconsin Hospital is a referral institution, clinical

diagnoses were available prior to cell collection for most patients used in the study. These diagnoses served as a basis for analysis of the data.

The cell samples were obtained at the time of speculum examination by instillation and aspiration of 5 ml. of Eagle's Minimum Essential Medium containing 10% fetal calf serum supplemented with penicillin and streptomycin. The cell collections were obtained with a Pipette fitted with a 5-ml, rubber bulb (Fig. 1).

Fig. 1. ***Tube containing Eagle's medium and pipette fitted with 5-ml. bulb, used to obtain cell samples for size distribution analysis. (Bulb from the Bellco Glass Co., Vineland, N. J.)***

Retrieval of the medium from the vagina generally yielded approximately two to ten million desquamated cells. If the sample contained mucus, it was filtered through cheesecloth prior to processing. The collected cell samples were reagitated and diluted 1:50 with particle-free physiologic saline solution for counting and volume distribution analysis. In this study, all samples were analyzed within three hours of collection.

Cell volume distribution pattern analyses were obtained by means of a model B Coulter Counter fitted with a 100-micron sampling aperture and a model H size distribution plotter. The theory and operation of the Coulter Counter have been described elsewhere.[1,10,15] The Coulter Counter was calibrated with ragweed pollen of known mean particle volume. Total cell counts were made at the setting having a volume range of 0 to 1,600 cup, using a lower threshold setting of 5 to screen out smaller debris and electronic noise, with the upper threshold disengaged. All volume distribution analyses were performed in duplicate: one at counter settings providing a volume distribution range of 0 to 1,600 cup; the other at a setting with a volume distribution range of 0 to 800 cup. For each patient, the collection of vaginal cells was preceded by acquisition of a smear which was analyzed in the Cytology Unit of the Wisconsin State Laboratory of Hygiene and reported as negative, atypical, suspicious, or positive. Patients were considered normal if they were asymptomatic and had normal cytology or negative biopsy findings, or both. Dysplasia, carcinoma *in situ*, and invasive carcinoma were diagnosed by tissue examination. No patient who had had a biopsy or curettage within a month prior to cell collection was included in the series.

In this series a volume distribution pattern was considered abnormal if: (1) the population of large cells consisted of two or more peaks appearing after window 4 at the 0 to 1,600 cu.$_{\mu}$ setting; (2) the population was present at the appropriate windows at a setting encompassing the 0 to 800 cu.$_{\mu}$ range (for example, if the peak of the second population falls in window 8 at the 0 to 1,600 cu.$_{\mu}$ setting, it must fall at approximately windows 15 or 16 at 0 to 800 cu.$_{\mu}$ setting, and must contain twice the number of peaks at the latter setting as at the former); (3) the population was reproducible on an additional printout from the same sample.

Trichomonas Vaginalis Cultures

Vaginal pool samples from selected patients, proven by wet smear to have Trichomonas vaginitis, were collected, using a pipette. The samples were inoculated into 7 ml. of the liver extract culture medium of Feinberg and Wittington,[5] and incubated at 37 C. in an atmosphere of 95% air and 5% CO_2. Subcultures were made every three to four days after microscopic examination to ascertain viability.. After seven to ten days of

culture, size distributions of *T. vaginalis* were determined. At that time, the average cell count was 1.5 to 2.0 x 10^6 viable organisms per ml.

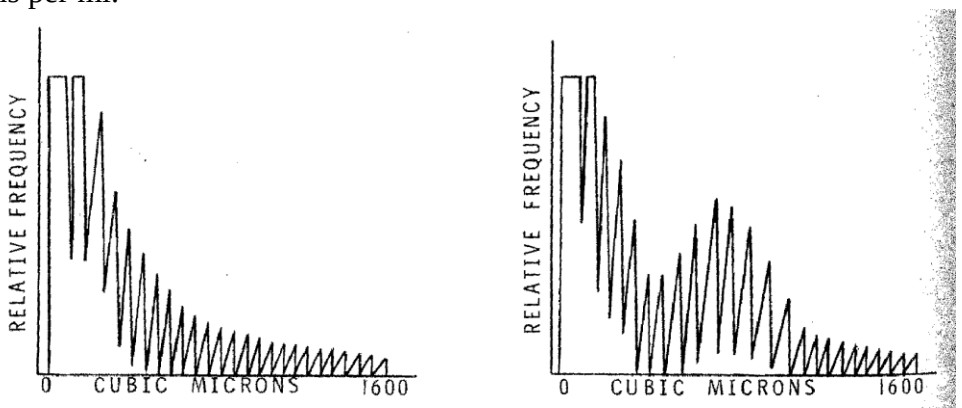

FIG. 2. Size distribution patterns printed out directly by the automatic plotter. Left, characteristic negative pattern obtained from a normal patient. Right, characteristic positive pattern from a patient with squamous-cell carcinoma of the cervix. Volume range 0 to 1,600 cu.μ. Volume increases from left to right.

Results

Figure 2 illustrates two patterns as they were printed out by the size distribution plotter. On the left is a characteristic negative pattern from a normal patient. On the right is a characteristic positive pattern from a patient with invasive squamous-cell carcinoma of the cervix. In both patterns each peak represents the relative number of cells in that particular volume range. The cell volume increases from left to right.

The patients studied totalled 339. As seen in Table 1, of 108 patients with negative smears, 75 had negative size distribution patterns and 33 had false-positive or abnormal size distribution patterns. However, 19 cases were unsatisfactory with the conventional smear due to insufficient numbers of cells, but were satisfactory for cell sizing because, with the latter technic, the cells are in suspension and the dilution factor for any particular specimen can be altered to obtain an adequate cell number for analysis, which is not possible with cell smears. An additional 16 patients (Table 1) with abnormal smears were found to have negative tissue findings. Of these 16, 12 had normal size distribution patterns and were classed as atypical with conventional cytology, and four patients had positive size distribution patterns. Three of these four had positive smears and one was classed as suspicious.

Table 1. Results of Volume Distribution Studies and Simultaneous Papanicolaou Smears for 124 Normal Patients

	Volume Distribution		Papanicolaou Smear					
	Positive	Negative	Positive	Suspicious	Atypical	Negative	Unsatisfactory	Number of Patients
Negative Papanicolaou smears								
Premenopausal	17	51	—	—	—	58	10	68
Postmenopaus l	14	17	—	—	—	23	8	31
Pregnant	2	7	—	—	—	8	1	9
TOTAL	33	75	0	0	0	89	19	108
Positive Papanicolaou smears with negative biopsies	4	12	3	1	12	0	0	16
TOTAL	37	87	3	1	12	89	19	124

Of 60 women shown to have dysplasia or carcinoma *in situ* confirmed by tissue examination, 40 had positive or abnormal top size distribution patterns (Table 2). smears made at the time of cell collection revealed 25 positive, seven suspicious, 12 atypical, and 15 false-negative smears, and one unsatisfactory smear.
When dysplasia and carcinoma *in situ* were analyzed separately, 12 of the patients (85.7%) with dysplasia were detected by cell sizing, whereas the conventional technic found two positive, three suspicious, four atypical, and five negative. Of the 46 patients with carcinoma in *situ,* cell sizing was positive in 28 cases, whereas conventional cytology scored 23 positive, four suspicious, eight atypical, 10 negative; in one case an unsatisfactory smear was reported.

Table 2. Results of Volume Distribution Studies of 60 Histologically Confirmed Intraepithelial Lesions

Lesion	Volume Distribution		Papanicolaou Smear					Number of Patients
	Positive	Negative	Positive	Suspicious	Atypical	Negative	Unsatisfactory	
Dysplasia	12	2	2	3	4	5	0	14
Carcinoma *in situ*	28	18	23	4	8	10	1	46
TOTAL	40	20	25	7	12	15	1	60

Of 96 patients (Table 3) with invasive squamous-cell carcinoma of the cervix, 82 patients (85.4%) were positive by cell sizing. The smear scored 62 of the 96 patients positive, eight suspicious, five atypical, and 13 negative. In eight cases, no smears were obtained at the time of cell collection. The 16 patients with squamous-cell carcinoma of the vagina and carcinoma of the ovary were obtained only incidentally. One might anticipate that the smear would not show any appreciable degree of accuracy in the detection of these lesions, because the smear samples are obtained by Ayre scrape of the cervix.

Table 3. Results of Volume Distribution Studies of 112 Histologically Confirmed Carcinomas

Lesion	Volume Distribution		Papanicolaou Smear						Number of Patients
	Positive	Negative	Positive	Suspicious	Atypical	Negative	N.A.*	Unsatisfactory	
Squamous-cell carcinoma of cervix	82	14	62	8	5	13	8	0	96
Ovarian	4	4	0	0	1	5	1	1	8
Vaginal	4	4	4	0	1	2	1	0	8
TOTAL	90	22	66	8	7	20	10	1	112

* Not ascertained.

Carcinoma of the ovary is thought to exfoliate only minimally, if at all, and it would seem unlikely for an appreciable number of cells to be obtained by cervical scraping. However, washing the vagina would increase the probability of collecting these cells if they were present. Surprisingly, the cell sizing technic detected only half the vaginal lesions, whereas the scrape did better. However, the cell sizing technic detected half the ovarian tumors, whereas the scrape missed all but one.

There were 54 patients with the histologic diagnosis of adenocarcinoma of the endometrium (Table 4); of these, 50 (92.8%) had positive size distribution patterns, and four (7.2%) were negative. By the conventional smear 15 were positive, five suspicious, five atypical, and 22 negative, with seven not ascertained.

Of five patients confirmed by tissue examination to have adenocarcinoma of the endocervix, all had abnormal size distribution patterns, and one was detected by conventional cytology. When the two adenocarcinoma categories are combined, size distributions were positive in 55 of 59 cases (93.2%), whereas the smear detected 26 of the 59 cases (44.4%).

Table 4. Results of Volume Distribution Studies and Simultaneous Papanicolaou Smears of 59 Histologically Confirmed Adenocarcinomas

Lesion	Volume Distribution		Papanicolaou Smear					Number of Patients
	Positive	Negative	Positive	Suspicious	Atypical	Negative	N.A.*	
Adenocarcinoma of the endocervix	5	0	1	0	0	4	0	5
Adenocarcinoma of the endometrium	50	4	15	5	5	22	7	54
TOTAL	55	4	16	5	5	26	7	59

* Not ascertained.

Trichomonas Vaginalis as an Interfering Factor

It was found in this study that when *T. vaginalis* organisms were present in vaginal washings, in some cases size distribution printouts similar to those which occur with uterine epithelial abnormalities were obtained.

As can be seen in Figure 3A, the size distribution of a pure culture of *T. vaginalis* grown at pH 6.5 shows a unimodal population with a modal volume of 600 cu.$_{\mu}$. If a sample from a normal patient (Fig. 3B) is seeded with 1.0 to 1.5 x 10^6 organisms, it can easily be seen (Fig. 3C) that the size distribution pattern coincides with that of an abnormal cell population. It has been well established that the sizes of the Trichomonas organisms can increase or decrease with changes in pH,[11] so that in suitable instances the volumes of the organisms are small enough not to interfere with the abnormal cell patterns (Fig. 4).

Viability of *T. vaginalis in vitro* is dependent not only on pH, but also on the presence of liver extract and certain other reagents in the medium. Therefore, if the pH of the collection medium (Eagle's medium with 10% fetal calf serum) is above pH 7.0 (Fig. 4A), and the organisms have been exposed to this medium for more than 20 hr., the volume of the organisms is such that they are beyond the volume range of the abnormal cell population. In the pH, range 7.0 to 8.0, if the Trichomonads are in Eagle's medium less than 20 hr. (Fig. 4B), they may appear as a unimodal population peaking at 500 to 600 cu.$_{\mu}$ (Fig. 4C). It is within this pH range that the volumes of the organism coincide with that of the abnormal cell population and serve as an interfering factor in the system. Therefore, by having the collection medium above pH 7.0 or by analyzing the samples after 20 hr., *Trichomonas vaginalis* can be greatly reduced as an interfering factor.

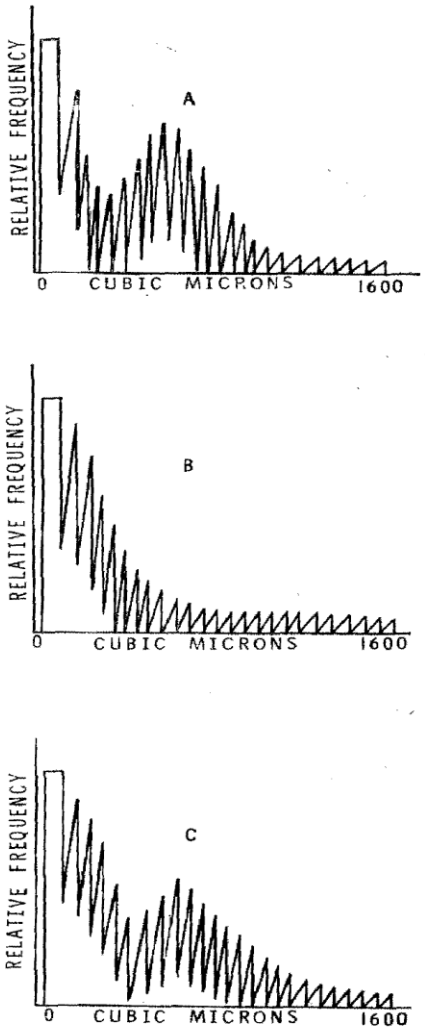

FIG. 3. Size distribution curves with *Trichomonas vaginalis*. (A) Pure culture of *T. vaginalis* grown at pH 6.5. Note unimodal population in range of 600 cu.μ. (B) Size distribution pattern of vaginal cells from a normal patient. No population of large cells can be seen. (C) Size distribution pattern of vaginal cells from normal patient seeded with *T. vaginalis* grown at pH 6.5.

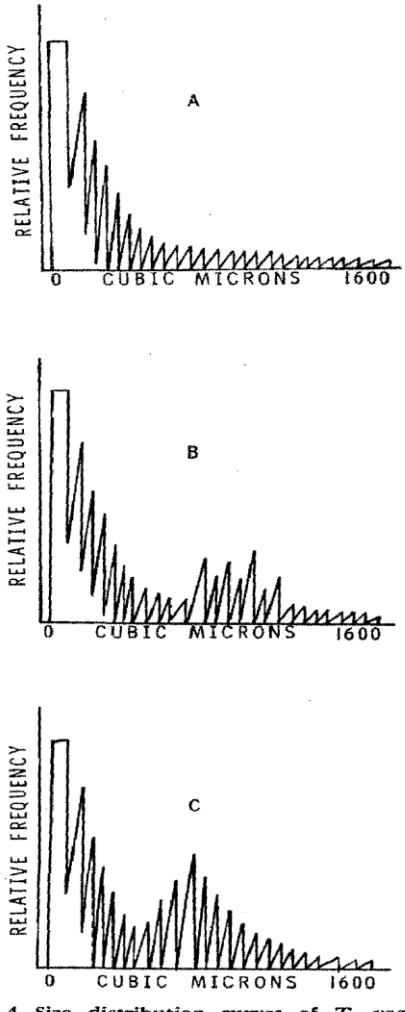

FIG. 4. Size distribution curves of *T. vaginalis*. (A) Pattern after incubation in Eagle's medium at pH 7.0 for 20 hours. No population of large cells is seen. (B) Pattern after incubation in Eagle's medium at pH 7.5 for 4 hours. Note the broad, irregular population in the range of 1,000 cu.μ. (C) Pattern after incubation in Eagle's medium at pH 6.5 after 4 hours. Note unimodal population in the range of 600 cu.μ.

Discussion

The data suggest that cell sizing may provide a basis for a rapid method of detection of uterine malignancy. With minimal processing of the specimen after col. lection, the results can be obtained in approximately 200 sec.

The high false-positive rate for cell sizing in this series may be partially explained by Trichomonas contamination. During *in vitro* studies with *T. vaginalis,* carried out simultaneously with this study and reported here, it was found that altering the pH of the collection medium can essentially eliminate Trichomonas contamination. When the pH of the collection medium was between 4.0 and 9.5, there was no change in the size distribution of the abnormal cell population present in vaginal washings collected from patients with squamous-cell carcinoma of the cervix. In a subsequent field trial of cell sizing[12] in which this factor was controlled, it was found that the false-positive rate due to *T. vaginalis* group decreased from 25% to 2%.

Of the 108 patients categorized as normal in Table 1, none had histologic confirmation. Therefore, it is conceivable that a portion of those categorized as cell size false-positive may in fact have some abnormality, but no verification was available.

In considering the false-negative cases, it appears that repeated collections will reduce their importance, because successive negatives probably will not occur in the presence of significant abnormalities. Furthermore, the prolonged incipiency of *in situ* or lesser lesions affords time for such repetition. The present data, although from a limited number of patients, also indicate that the number of false negatives encountered with cell sizing is about the same as that with the cervical scrape.

In the tables, the smears were classified as "negative," "atypical," “suspicious,” or "positive." While the “suspicious” and "positive" categories indicate the presence of cells thought to have arisen from a malignant lesion, the "atypical" category does not.

The significance of the atypical smear depends upon the cytologic interpretation by the individual laboratory. In this laboratory the atypical classification is used to denote slight cytoplasmic or nuclear alterations in cellular morphology, associated in the majority of cases with inflammatory reactions such as cervicitis, vaginitis, or other benign conditions. This definition is shared by Taylor,[18] Slate,[18] Koss,[9] and Hulka.[7] Following a reading of "atypical," a repeat procedure is usually requested. Thus, it is difficult to determine whether the "atypical" classification should be included as a negative or classed with the positive categories when comparing two technics as to their accuracy in detecting malignancy. This ambiguity is a significant factor since the size distribution results are classified as either "positive" or "negative" and the relative sensitivity of the smear in detecting cervical abnormalities in relation to that of the size distribution method can be made more or less favorable depending on the disposition of the “atypical” category.

The results of this study show that cell sixing efficiently detects preinvasive lesions of the uterus. This is evidenced by both histologic verification and correlation with the conventional smear. For detecting invasive squamous-cell carcinoma of the cervix, the two technics were about equally sensitive. However, for detecting adenocarcinoma of the endocervix and endometrium, cell sizing appeared to be more than twice as sensitive as the Ayre scrape.

After the publication of the initial study of University of Wisconsin Hospitals patients screened by cell sizing,[13] the technic was attempted in other laboratories.

Valliant and Richart [20] applied the technic of cell sizing to 65 women with cytologically established carcinoma *in situ* and dysplasia. Their patient sample was selected from those patients found on previous examination to have abnormal smears. Any patient who had negative cytology at the time of cell collection was excluded from the study. The cell collections were obtained by a physician using a Davis irrigation device [2] containing Eagle's

Minimum Essential Medium with penicillin and streptomycin. No serum, an ingredient found by us to be necessary for cell volume stability, was used in the medium.

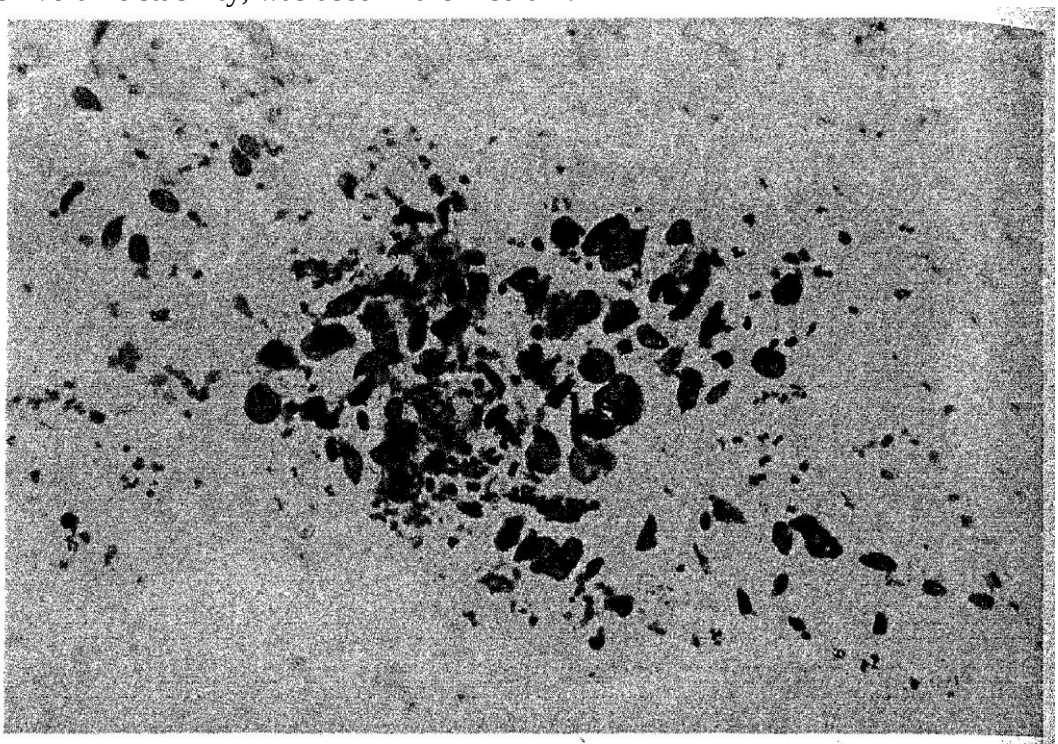

FIG. 5. Photomicrograph of malignant cells from a patient, with a positive size distribution pattern. Note malignant cells are fairly well separated. Some clumping occurs in the preparation of the smear. Papanicolaou stain. × 300.

Valliant arid Richart[20] also found, in a series of ten patients, that the volume distribution of peripheral leukocytes was identical with the bimodal distribution of abnormal vaginal cell collections. Investigating this fact, they incubated both leukocytes and vaginal washings with Triton X-100, a stromatolyzing agent, and found that the second large cell population disappeared. They concluded that this large cell population, therefore, must have been leukocytes and not neoplastic cells. In an attempt to verify their findings, we obtained Triton X-100 from these authors and found that Triton X-I00 caused the malignant cells to dump, forming a clot of the cell suspension so that no epithelial cells were able to enter the aperture of the Coulter Counter (Figs. 5 to 7). Understandably, then, no second large cell populations were evident on the size distribution patterns.

The fact that these authors found peripheral leukocytes collected in heparin to have the same size distribution pattern as the abnormal cells from vaginal washings is not in agreement with the findings of Ladinsky and Westrincg.[14] In a series of studies comparing normal and leukemic leukocytes obtained in a series of anti-coagulants, it was found that peripheral Leukocytes obtained from normal subjects and those from patients with various solid tumors had a modal volume of 210 cu. μ. This is much smaller than the modal volumes of the abnormal cells found in patients with uterine abnormalities. These findings were corroborated by Gauthier and Harrel.[6] This difference in modal volume is great enough that leukocytes, in most cases, were found not to be interfering factors.

It is interesting that just prior to their publication on cell sizing, Richart and Valliant[17] published a report on the Davis irrigation smear, in which they found a 50% false-negative rate for "presumptive" (cytologically proven) carcinoma *in situ,* a 51% false-negative rate for presumptive dysplasia, and a 39% (5 of 13) false-negative rate for histologically-proven carcinoma *in situ.* Despite the high false-negative rate found with the Davis irrigation device, these authors used the same device to evaluate cell sizing and found a 44% false-negative rate for cytologically-proven carcinoma *in situ,* a 60% false-negative rate for cytologically-proven dysplasia, and a 35% false-negative rate for histologically proven carcinoma *in situ.* In this study, of 274 patients classified as having carcinoma in *situ* and dysplasia, only 13 cases were histologically confirmed. The remaining 261 were diagnosed solely on the basis of the, smears, Therefore, there is a question whether these lesions were, in fact,

present; few laboratories are able to characterize the nature of a preinvasive lesion exactly on the basis of the smear alone.

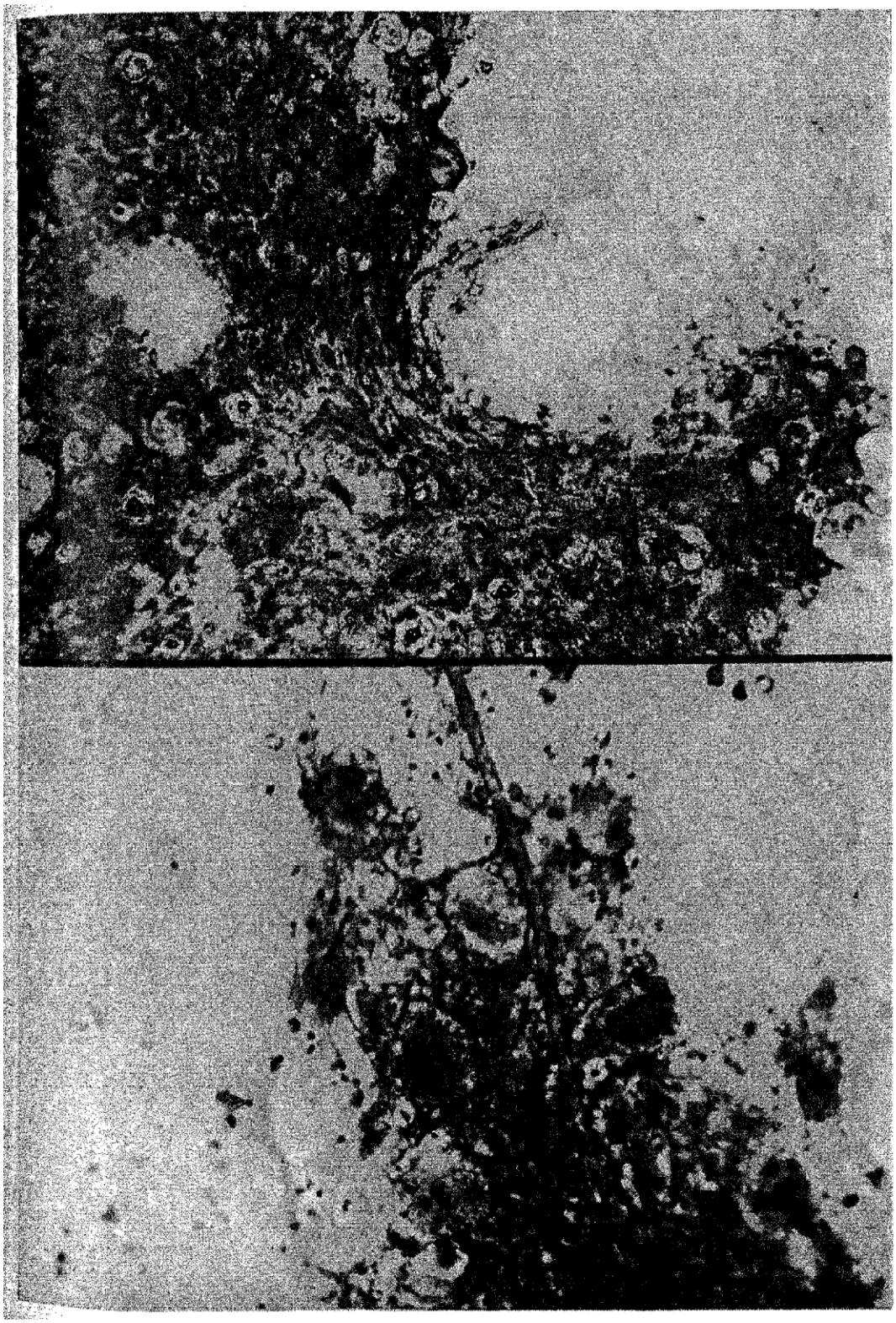

Fig. 6 (upper). ***Photomicrograph of a clot formed after cell suspension is exposed to Triton X-100 for 10 minutes. Note malignant cells surrounded by amorphous colloidal matter. stain. x 200.***
Fig. 7 (lower). ***Higher-power view of clot formed by Triton X-100. Note malignant cells emersed in colloidal matter. stain. x 300.***

It may be concluded that the Valliant and Richart study was not a repetition of our initial study, since the technics of collection and analysis were different and the data were, at best, equivocal.

In 1966, Iverson and associates[8] also attempted to test the method of cell sizing. However, these authors used an 0.3 M solution of isethionic acid as the collection fluid. A 10 ml. syringe furnished with a 4-inch, 16-gauge needle was placed 1 inch from the external os and under maximum pressure the fluid was instilled and aspirated from the posterior fornix. This procedure was repeated three times for each cell collection. These authors found, on screening 30 patients with carcinoma of the cervix, that 19 were positive (63.3%), and 11 were negative (36.7%). They also state that the "Coulter negative washings contained a large number of erythrocytes—far in excess of any epithelial cell type." It is understandable that the harsh method of cell collection used by these authors caused bleeding which contaminated the "Coulter negative washings" excessively with erythrocytes.

In an attempt to verify the findings of these authors, we analyzed duplicate washings from patients known to have invasive carcinoma of the cervix. One was collected and analyzed in tissue culture medium and the other in isethionic acid. In all cases, the second large cell population was found to be present in the specimens in which the cells were suspended in tissue culture medium and absent in the washings containing isethionic acid. It must be concluded that the Iverson study was not a repetition of our technic of cell sizing as originally published.

On the other hand, Perez-Mesa and coworkers[16] made an independent study of cell sizing of 100 patients following our original method. They found 91% agreement between the smear interpretation and the size distribution curves. No false-normal curves were obtained. The false positives encountered were those associated with trichomoniasis.

H. Davis in collaboration with A, Stafl,[4] after initial consultation with this laboratory, also attempted a corroborative study of cell sizing. Their patient sample included 34 patients with carcinoma *in situ,* 30 patients with invasive squamous-cell carcinoma of the cervix, and ten patients with adenocarcinoma of the endometrium. Their findings on cell sizing were: 79% accuracy for carcinoma *in situ,* 93% accuracy for invasive squamous-cell carcinoma of the cervix, and 90% accuracy for adenocarcinoma of the endometrium. Concerning those patients with carcinoma in *situ,* Davis[3] states that his classification is approximately equivalent to a diagnosis of dysplasia by most other pathologists.

From the four studies described above in which the cell sizing method has been tested as a means for the detection of uterine cancer, the results from the first two can be questioned, since the methodology was in no way similar to the one used by us. The studies of Perez-Mesa and Davis, on the other hand, must be considered independent evaluations of the technic. Their results, although preliminary in that the patient samples were small, appear to corroborate the results of our study.

The present findings indicate that cell volume distribution pattern analysis may prove useful as a mass prescreening technic. However, the patients used in this study, for the most part, had been diagnosed prior to the cell collection. To further evaluate the technic of cell sizing as a mass prescreening method, a limited field trial was undertaken in which 1,750 asymptomatic patients were screened. The results of the field trial are currently being ana1yied.[12]

The advantages of cell sizing as a prescreening technic are: (1) the Coulter counter is commercially available, and its operation can be mastered by untrained personnel in a matter of days because the technic depends on semiautomated cell volume analysis rather than morphology; (2) the time involved in the preparation, analysis, and interpretation of the specimen is approximately 3 min.; (3) the elimination of the vast majority of negative patients now visually screened by conventional cytology would permit more effective use of trained cytotechnologists for screening of abnormal cytology. These studies suggest that the false-positive rate is acceptable for a screening technic and the false-negative rate may fall in the same range as that of the smear.

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